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Molecular Detection and Characterization of Yellow Fever Virus in Blood and Liver Specimens of a Non-Vaccinated Fatal Human Case

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A yellow fever virus of a South American genotype was identified in the liver and blood samples of a non-vaccinated European patient after his return from Brazil. ELISA tests were negative for IgG and positive for IgM against yellow fever. Yellow fever proteins in the formalin-fixed and paraffin-embedded liver biopsy were detected by immunohistochemical procedures. Viral RNA extracted from the liver tissue was also detected using an RT-semi-nested PCR procedure and molecular hybridization. Alignment of the sequence obtained from a gene fragment amplified by RT-semi-nested PCR directly from a blood sample with those of African and South American yellow fever virus strains identified a Brazilian topotype as being responsible for the disease. RT-semi-nested PCR may be used advantageously for clinical specimens for rapid and specific diagnosis, and with archival biopsy material for retrospective studies. ***J. Med. Virol. 53:212–217, 1997.***

KEY WORDS: yellow fever virus, liver, blood, immunohistochemistry, RT-PCR, sequence

INTRODUCTION

Yellow fever virus belongs to the *Flaviviridae* family, flavivirus genus [Monath and Heinz, 1996]. Yellow fever is an acute viral disease which is endemic in tropical areas of Africa and the Americas [Digoutte et al., 1995]. Yellow fever has two types of transmission cycle: urban and sylvatic. It can be transmitted from infected humans to susceptible humans by the urban mosquito vector *Aedes aegypti* and incidentally from infected non-human primates to humans by sylvatic mosquito vectors. Clinical forms range from an asymptomatic form or mild febrile illness to a fulminant fatal infection [Digoutte et al., 1995]. The fatality rate in severe cases is between 20% and 50%. Within the last three years, there have been yellow fever outbreaks in Nigeria, Ghana, Kenya, Liberia, Sierra Leone, Cameroun, Gabon and in Peru [Robertson et al., 1996]. The re-emergence of yellow fever seems to be due largely to the lack of a follow-up strategy for mass vaccination and the collapse of the global effort to control *Aedes aegypti*. [Robertson et al., 1996]. Tourists and travellers in zones in which yellow fever is endemic can be infected by mosquito bite and then die from yellow fever after returning to their home countries. In France, two tourists died in 1979 after a short trip to southern Senegal [Digoutte et al., 1991]; and in 1996 two travellers returning from Brazil also died [Barros and Boecken, 1996, Anonymous, 1996].

It is difficult to diagnose yellow fever in the presence of fever and jaundice and to differentiate it from viral hepatitis, other arboviral and haemorrhagic fevers, bacterial and parasitic diseases, and from diseases due to toxic substances. The virus is generally not detected in the blood during the late phase of the disease due to an increase in the neutralizing antibody titre. Immunohistological analysis of liver biopsies [Monath et al., 1989] and molecular detection of viral RNA in blood samples [Deubel and Pierre, 1994] are sensitive and specific tests to identify yellow fever virus. We investigated a case of yellow fever in previously healthy tourist who travelled to the Amazon region in Brazil and died two days after his return to Switzerland [Barros and Boecken, 1996]. Diagnosis of yellow fever was established by immunohistological analysis of post-

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mortem liver samples. Yellow fever RNA was identified in serum and liver samples by hybridization and direct sequencing of an amplified viral gene fragment. The results demonstrate the potential value of RT-PCR for rapid diagnosis and epidemiological investigation.

MATERIALS AND METHODS

Biological Material

Samples from a 54 year-old male (R.A.) who died in April 1996 in Basel, Switzerland, after a journey to Brazil, were sent to the WHO Collaborative Centre for Research on Arboviruses and Viral Haemorrhagic Fevers at the Pasteur Institute in Paris. Serum collected one day before his death and formalin-fixed paraffin-embedded liver tissue were studied. IgM and IgG specific for flaviviruses were determined in the serum using ELISA tests described previously (Lhuillier and Sarthou, 1983). The clinical diagnosis of suspected yellow fever was supported by histopathological examination of the liver at the Institute for Pathology in Basel.

Cell Culture Analysis

*Aedes pseudoscutellaris AP*61 mosquito cells were grown in Leibovitz L15 medium containing 10% tryptose phosphate, 10% fetal calf serum (FCS), and antibiotics. Subconfluent cells were incubated for one hour with a 1 in 10 dilution of serum sample in L15, washed, and incubated for seven days in L15 medium containing 5% FCS. Viral antigens were detected by indirect immunofluorescence assay (IFA) using anti-yellow fever hyperimmunized mouse ascitic fluid (HMAF) and fluorescein-conjugated anti-mouse IgG (Biosys).

Molecular Diagnosis

RNA from serum samples was extracted using a modification of a procedure described previously [Deubel et al., 1990]. Ten μl of serum sample were diluted 1/5 in TE buffer and mixed with 50 μl of lysis buffer (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, 100 mM β-mercaptoethanol) and 150 μl of a phenol-chloroform mixture. RNA was precipitated using an equal volume of isopropanol. RNA was extracted from a 5-μm section of a paraffin-embedded block of liver. A surface area of 5–10 mm^2 of tissue was scraped off the slide, transferred to an Eppendorf tube, and deparaffinized with 400 μl of xylene. The sample was centrifuged at 10,000 rpm for 5 min and the pellet was washed with 400 μl of ethanol, and air-dried. Subsequently, the pellet was resuspended in 100 μl of TE buffer-0.5% SDS and treated at 56°C for 1 hr with proteinase K (Boeringer–Mannheim, 1 mg/ml) in the presence of 20 units of RNasin (human placental ribonuclease inhibitor, Promega). Finally, the RNA was extracted with 100 μl of phenol-chloroform, and chloroform, and precipitated with two volumes of ethanol in 0.3 M ammonium acetate.

RT-PCR was carried out as described previously [Pierre et al., 1994]. Briefly, 1 μl (30 ng) of primer VD8 (5′-GGGTCTCCTCTAACCTCTAG-3′) was mixed with RNA resuspended in 10 μl of distilled water and the mixture was heated at 95°C for 2 min and placed on ice. cDNA was synthesized in RT incubation buffer (provided by the manufacturer), 0.2 mM of each of the four triphosphate deoxynucleotides (dNTPs), 20 units RNasine and 2 units of avian myeloblastosis virus reverse transcriptase (Boehringer–Mannheim) by incubation for 1 hr at 42°C. cDNA was amplified by PCR. Four μl of the cDNA were added to 46 μl of a mixture containing *Thermus aquaticus* (*Taq*) polymerase buffer (provided by the manufacturer), 2 mM $MgCl_2$, 0.5 mM of each of the four dNTPs, 300 ng of primer VD8 and of degenerated primer EMF1 (5′-TGGATGACSACKGARGAYATG-3′) (S = C,G; K = G,T; R = A,G; Y = C,T), and 0.5 unit of *Taq* polymerase (Perkin Elmer Cetus). After 5 min denaturation at 95°C, the mixture was subjected to 30 PCR cycles: 95°C for 30 sec, 53°C for 90 sec and 72°C for 60 sec followed by a final 10-min polymerization step at 72°C. One two-hundredth of the PCR product was used for semi-nested PCR using primers VD8 and NS5YF (5′-ATGCAGGACAAGACAATGGT-3′). After the denaturation step, DNA was amplified by 25 cycles of PCR: 94°C for 30 sec, 55°C for 60 sec, and 72°C for 120 sec, followed by a final extension step at 72°C. Negative controls were included in the series which consisted of serum from an healthy individual. The positive control consisting of RNA extract from yellow fever virus-infected mosquito cells (Pierre et al., 1994) was tested separately to avoid any contamination and was not included in the series.

Analysis of PCR Products

The amplicons were confirmed by dot-blot hybridization as previously described using a yellow fever DNA probe [Deubel et al., 1990]. The probe was synthesized by PCR using VD8 and NS5YF primers and a recombinant plasmid containing a French neurotropic yellow fever virus sequence framed by VD8 and EMF1 sequences [Pierre et al., 1994]. The probe produced by PCR was purified by ion exchange chromatography (Wizard, Promega) and 0.2 μg was labelled with ^{33}P-α-dATP (Amersham) by nick-translation (Boehringer-Mannheim). Half the amplicon products obtained from the first PCR run and from semi-nested PCR were denatured in NaOH [Deubel, et al., 1990], and dotted onto a nitrocellulose membrane. The membrane was pretreated in hybridization buffer (Amersham) and incubated overnight at 45°C in the same buffer containing the labelled probe. After washing at 65°C, the membrane was exposed to X-ray film. Half of the semi-nested PCR product obtained from the serum sample was purified on a low melting-point agarose gel in TAE buffer and sequenced by the dideoxynucleotide method using either VD8 or NS5YF primers and *Taq* polymerase, according to the instructions of the manufacturer (Biolabs).

Immunohistological Study

Sections of paraffin-embedded liver were deparaffinized with xylene, rehydrated with a series of alcohol solutions, each diluted successively, treated with pro-

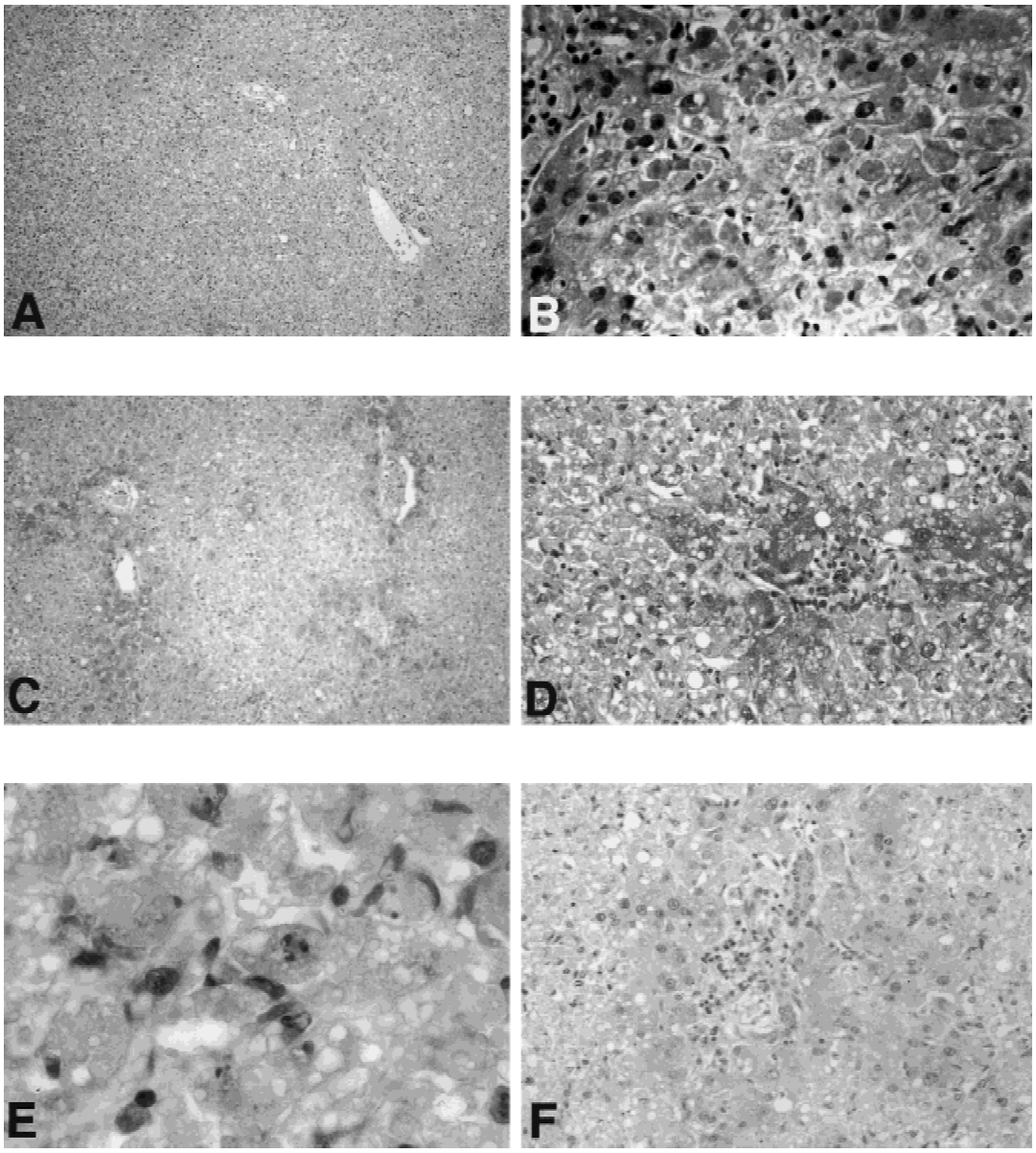

Fig. 1. Histological examination of liver section from yellow fever case RA/96. A) hematoxylin-eosin staining showing midzonal necrosis, fatty metamorphosis and eosinophilic degeneration consistent with the diagnosis of yellow fever. Hematoxylin-eosin (HE) ×100. B) Eosinophilic degeneration: Councilman bodies. HE ×250. C) Immunohistochemistry using an anti-yellow fever antibody A.P.A.A.P. method and fast red. Positive signal observed in the cytoplasm of hepatocytes are in periportal areas and around necrosis. ×250. D) Immunohistochemistry with anti-yellow fever antibody-A.P.A.A.P. ×400. Hepatocytes containing large amount of yellow fever antigens. E) Immunohistochemistry with anti-yellow fever antibody-A.P.A.A.P. ×1000. F) Immunohistochemistry using anti-dengue fever antibody-A.P.A.A.P. ×250. Negative hepatocytes.

teinase K and incubated with anti-yellow fever or anti-dengue 2 HMAF and 5E3 yellow fever anti-E MAb (kindly provided by J. J. Schlesinger). Antibody binding was visualized by the alkaline phosphatase-anti-alkaline phosphatase technique (A.P.A.A.P.) as previously described [Monath et al., 1989, Polak and Nordeen, 1986].

RESULTS

Histology and Immunochemistry

The serum sample contained IgM but not IgG that reacted with yellow fever antigens (data not shown). This suggested that the patient may have contracted yellow fever during his journey to Manaus, Brazil. There were characteristic histopathological changes compatible with the diagnosis of yellow fever. The three diagnostic markers were observed: (1) incomplete necrosis of parenchyma with a small rim of a few viable hepatocytes around the portal tracts which may be consistent with an extended form of midzonal necrosis; (2) centrilobular cells with fine fat vacuolization and multiple and microvacuolar changes; (3) eosinophilic degeneration with sharply defined, round, eosinophilic,

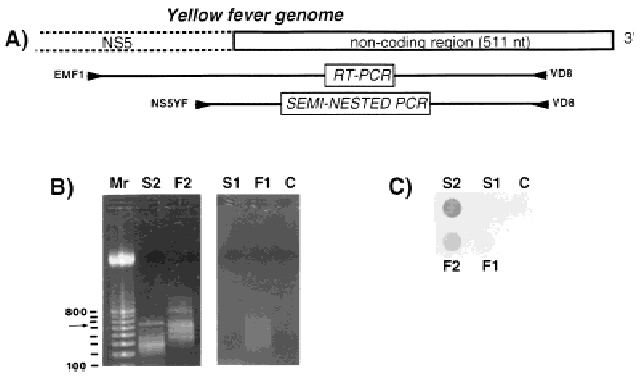

Fig. 2. Identification of RA/98 yellow fever virus products amplified by RT-semi-nested PCR of RNA extracted from serum and liver biopsy. A) schematic representation of the amplification procedure. Oligonucleotide primers EMF1, NS5YF and VD8 are described in Materials and Methods. B) Identity of amplified products on ethidium bromide-stained 2% agarose gel. RNA extracted from serum (S) or from liver (F) of the patient, and from serum from a healthy individual (C) was subjected to RT-PCR (1) and semi-nested PCR (2). The expected yellow fever amplified product of 542 nucleotides is indicated by an arrow. C) Dot-hybridization assay of the amplified products using yellow fever-specific DNA probe. The ^{33}P-labelled probe corresponded to VD8-NS5YF-amplified product.

cytoplasmic structures termed Councilman or Councilman-like bodies (Fig. 1A–B). Using yellow fever polyclonal antibodies in HMAF and anti-E MAb a positive signal was detected in the cytoplasm of hepatocytes around the portal tracts as well as in sinusoidal and endothelial cells (Fig. 1C–E). Negative controls were included in this study: dengue HMAF did not label replicate sections of the liver (Fig. 1F); sections of liver from a patient with an unrelated viral disease were negative with yellow fever HMAF (data not shown).

Identification of Yellow Fever Virus in Serum and Fixed Liver Specimens by Semi-Nested PCR

Yellow fever virus could not be isolated by cell culture (data not shown). We developed recently an RT-PCR method as a possible alternative to mosquito cell culture for rapid and sensitive detection of flaviviruses in biological samples [Deubel and Pierre, 1994, Pierre, et al., 1994]. This technique uses consensus flavivirus sequences EMF1 and CS2 (to which VD8 is complementary) for RT-PCR coupled to molecular hybridization for specific identification of the amplified product (Fig. 2A). Using a single step PCR assay, no amplicon was detected after amplification of the serum sample and the liver specimen (Fig. 2B). To improve the sensitivity of the method, a semi-nested PCR was performed using an internal primer (NS5YF). This contains a sequence specific for yellow fever virus as determined by comparative sequence alignment of different flaviviruses [Pierre et al., 1994]. The expected size of the amplicon produced by semi-nested PCR was 542 nucleotides based on the length of sequences published recently for South American yellow fever strains [Wang et al. 1996; Deubel and Drouet, 1997]. A band of the expected size was clearly visible after semi-nested PCR of serum extract (Fig. 2B). Several bands were visible after semi-nested PCR of the liver extract. However, one of the bands was of the expected size and may correspond to yellow fever-specific RNA. To investigate whether the amplicons were yellow fever-specific sequences, the amplified products were tested by dot-hybridization using a yellow fever specific probe. Amplicons obtained from serum and liver samples by semi-nested PCR hybridized with the probe whereas those obtained by RT-PCR did not (Fig. 2C).

Sequencing the Product Amplified by Semi-Nested PCR from the Serum Sample

The amplified DNA product of 542 nucleotides produced from the RNA in the patient's serum was purified and sequenced. The first 127 nucleotides of the 3′ non-coding sequence were aligned with those of the Asibi strain [Hahn et al., 1987] and a Brazilian isolate from 1935 [Wang et al., 1996] using the CLUSTAL V program [Higgins and Sharp, 1988] (Fig. 3). The sequence of the amplified gene differs from that of the older Brazilian strain at only five positions, suggesting a low rate of evolution during the 61 year period between the two isolations (6×10^{-4} changes per nucleotide per year). A deletion of 69 nucleotides was observed in the yellow fever-specific repeat sequences when compared to West African Asibi strain (Fig. 3).

DISCUSSION

Histopathological diagnosis is still used widely for yellow fever [Digoutte et al., 1995]. However, the causative agent remains uncertain and other approaches have been developed which involve immunoperoxidase or immunofluorescein staining, and nucleic acid hybridization to detect virus material in formalin-fixed liver sections [Monath et al., 1989, Hall et al., 1991, Barth et al., 1988]. In this study, we demonstrated the value of using immunocytochemistry in fixed human liver sections to detect the yellow fever antigen. The strong signal observed with monoclonal anti-envelope protein antibody confirmed the diagnosis suggested by the presence of anti-yellow fever IgM and conventional histology.

Unless serum samples from viraemic patients are preserved in good condition, i.e. in a refrigerator for a few days or at −80°C, it is very unlikely that yellow fever can be isolated by cell culture or by inoculating mice or mosquitoes [Digoutte et al., 1995]. In this patient, virus isolation failed. The serum we received was shipped at room temperature and this may explain why virus could not be isolated from this specimen. In addition, the presence of IgM in serum may have reduced the chance of isolating the yellow fever virus by classical mosquito cell culture.

We described previously an RT-PCR technique for rapid diagnosis of mosquito-borne flaviviruses using universal primers coupled to hybridization with specific molecular probes [Pierre et al., 1994]. This technique was tested further on human serum samples. It detected dengue viruses in IgM sera which scored

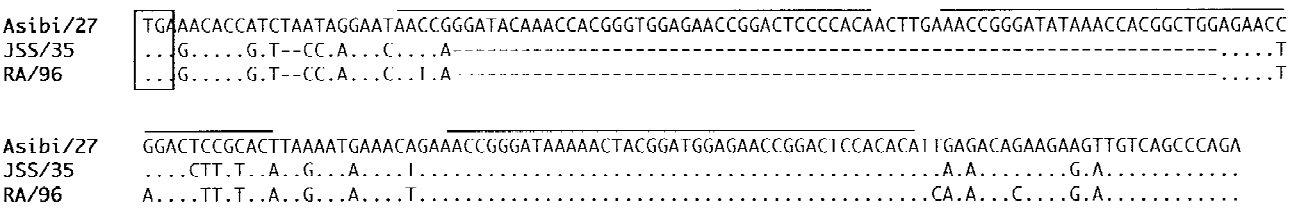

Fig. 3. Comparative aligned sequence of RA/96 (Brazil 1996) yellow fever amplified product with Asibi (Ghana 1927; Hahn, et al., 1987) and JSS/35 (Brazil 1935; Wang et al., 1996). Sequences start at the first nucleotides in the 3′ non-coding region downstream from the NS5 gene and TGA stop codon (boxed). The nucleotide identity with Asibi is indicated by dots and gaps in the alignment are indicated with dashes. Repeated sequences are overlined.

negative in cell culture [Deubel and Pierre, 1994]. In this study, the single step RT-PCR procedure failed to identify yellow fever virus RNA in blood and liver suggesting that there were only small amounts of viral material in the biological specimens. However, a study on dengue fever indicated that the dot-blot hybridization method was less sensitive than semi-nested PCR [Lanciotti et al., 1992]. To improve the sensitivity and specificity of our technique, semi-nested PCR was used. Several bands were observed in agarose gel after ethidium bromide staining, presumably due to non-specific binding of the primers to cellular nucleotide sequences or to jumps of the reverse transcriptase favoured by the substantial secondary structures of the 3′ non-coding region of the yellow fever virus genome [Kupiec and Sonogo, 1996]. Molecular hybridization with a yellow fever-specific probe and direct sequencing of the amplified product that was the expected size were also used (Figs. 2 and 3).

The 3′ non-coding region of the genome of the yellow fever virus is genetically diverse. In wild type and attenuated strains there are three repeated sequence elements in the 3′ non-coding region. They are 42 nucleotides long, and each differs from the others at four or five positions [Wang et al., 1996; Deubel and Drouet, 1997]. We demonstrated that the nucleotide sequence downstream of the NS5 stop codon in the 3′ non-coding region of the yellow fever virus was deleted in our Brazilian case (Fig. 3). Several strains from South America were recently shown to have similar deletions but with a certain degree of variability [Wang et al., 1996]. The similarity of the nucleotide sequence of the two Brazilian yellow fever viruses circulating in 1935 and 1996 suggests that there is slow evolution of this virus, presumably due to strong selective pressures maintaining the sequences of the viruses endemic in this ecological niche [Deubel et al., 1985]. Sequence determination directly from biological specimens is accurate as it avoids variant strain selection or mutation associated with serial passages of virus isolates. Paraffin sections from autopsy may be a good substrate for RT-PCR, and studies on archival formalin-fixed and paraffin-embedded liver samples may be useful for exploring retrospectively undiagnosed yellow fever cases and genetic characteristics of ancestor viruses.

Rapid diagnosis of yellow fever is critical, even after the death of the patient. An antigen capture technique was developed which detects directly yellow fever antigen in serum and liver [Monath and Nystrom, 1984]. This technique was slightly less sensitive than virus isolation. The sensitivity of semi-nested PCR was comparable to that of virus isolation [Lanciotti et al., 1992; Trent and Chang, 1992] and therefore, although more time-consuming than antigen capture, this approach has the advantage of sensitivity.

The etiology of many rapidly fatal diseases remains unknown, because there is a broad differential diagnosis for patients with signs and symptoms of yellow fever. Dengue and other hepatic and haemorrhagic viral diseases present very similarly to yellow fever. However, yellow fever should be suspected in any febrile patient who was recently in an area endemic for yellow fever and had not been vaccinated. However, the areas to which this patient travelled were not declared as endemic areas and a high degree of suspicion is essential to ultimately diagnose rare cases. In this study, we demonstrate that RT-semi-nested PCR is a powerful tool for yellow fever diagnosis, allowing improved surveillance and case notification to prevent spread of the disease.

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